Original Article

The Effect of Melatonin on The Developmental Potential and Implantation Rate of Mouse Embryos

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Abstract

Objective: Melatonin is a scavenger agent that has been used to promote *in vitro* embryo development. This study was designed to show the effects of melatonin on the quality and quantity rate of preimplantation mouse embryo development and pregnancy.

Materials and Methods: In this experimental study, super ovulated, mated mice were killed by cervical dislocation to collect two-cell zygotes from the oviduct of pregnant 1 day NMRI mice. Zygotes were cultured to the hatching blastocyst stage and the numbers of embryos at different stages were recorded under an inverted microscope. The cleavage rates of two-cell zygotes were assayed until the blastocyst and hatching blastocyst stage in drops of T6 medium that contained either melatonin (1, 10, and 100×10^{6}, 10 and 100×10^{9} M) or no melatonin. The cell numbers of blastocysts were determined by differential staining, implantation outcomes were studied, and development and pregnancy rate were compared by the Chi-square (development) and Fisher's exact (pregnancy rate) tests.

Results: The addition of 10 and 100 nM melatonin to the embryo culture media promoted the development of the two-cell stage embryos to blastocyst and hatching blastocysts ($p<0.01$) and caused a significant increase in total cell number (TCN), trophoectoderm (TE), and inner cell mass (ICM) of the blastocysts ($p<0.01$). A difference was observed in the percentage of transferred embryos that were successfully implanted between the control and treatment groups ($p<0.05$).

Conclusion: The data indicate that 10 and 100 nM of melatonin positively impact mouse embryo cleavage rates, blastocyst TCN, and their implantation. Therefore, melatonin at low concentrations promotes an embryonic culture system in mice.

Keywords: Development, Implantation, Melatonin, Differential Staining, Cleavage

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Introduction

The *in vitro* production (IVP) of mouse embryos is an important method for improving reproductive technologies and genetics. However, the developmental potential of embryos produced by IVP is still low, and optimization of embryo culture media would increase the production of developmentally competent embryos (1). The proliferation of fertilized eggs in culture conditions is arrested at the two-cell stage where free radicals are involved in the *in vitro* developmental block of two-cell embryos (2). The imbalance between the production of free radicals and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress (OS). Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (3). The effects of OS depend on the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe OS can cause cell death through necrosis, while even moderate oxidation can trigger apoptosis (4). Free radicals at physiological concentrations are also known to play a role in intracellular signaling, as it is involved in the normal processes of cell proliferation, differentiation, and migration (5). Even in the reproductive tract, free radicals play a dual role and can modulate various reproductive functions or lead to pathologies. Free radicals must be scavenged by antioxidants in the body. One of the antioxidants implicated to protect the body from free radicals is a hormone named melatonin (6). Melatonin is secreted by the pineal gland in the brain (7) and plays an important role in regulating the neuroendocrine system. This hormone is one of the major role players in the regulation of the circadian sleep-wake cycle. It is normally released from the pineal gland during the night in response to environmental changes in light levels (8).

The effect of melatonin on the *in vitro* developmental quality and quantity of mouse embryos, successful rate of embryo transfer, and subsequent pregnancy is not clearly elucidated. To evaluate the possible effect of melatonin on embryonic cleavage, developmental potential, blastocyst quality, and sequential embryo transfer, we have cultured mouse two-cell embryos in a development medium supplemented with different doses of melatonin until the blastocyst stage after which differential staining and embryo transfer were performed.

Materials and Methods

Animals

In this experimental study, a total of 35 female mice were housed individually in an air-conditioned room under a 12 hour light: 12 hours dark cycle (6 am: 6 pm), fed a commercial diet, and given water ad libitum. NMRI mice, 6-8 weeks old, were super ovulated by i.p. injection of 5 IU pregnant mare serum gonadotropin (PMSG; Organon, Holland) followed 48 hours later by an intraperitoneal (i.p.) injection of 5 IU human chorionic gonadotropin (hCG; Sigma, Germany). They were paired overnight with males of proven fertility. Two-cell embryos were mechanically obtained from their oviducts and collected in T6 medium and dishes that had been pre-warmed in an incubator at 37˚C.

All the animal experimentation in this study was approved by the Guilan University of Medical Sciences (GUMS) Animal Ethics Committee.

Animals

Mouse embryos at the two-cell stage were collected mechanically from oviducts of mated animals 46 hours after hCG injection. Embryos were collected and then washed three times in T6 medium (Sigma, Germany) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma, Germany).

Experimental group

To assess the effects of melatonin (Sigma, Germany) on *in vitro* embryo development, 10-15 embryos were cultured in 50 µl of the T6 medium that contained either 0, 10, and 100 nM, or 1, 10, and 100 µM of melatonin. The melatonin stock solution was prepared with an ethanol/T6 system as follows: 23.23 mg melatonin was first dissolved in 0.1 ml absolute ethanol and 9.9 ml of T6 medium which resulted in a 100 µM concentration, then serially diluted in T6 medium. In this manner, we prepared the 1, 10, and 100 µM and 10 and 100 nM melatonin stock solutions. The control group contained no melatonin, whereas the 0.1% ethanol media was the vehicle group. The stock solutions were stored refrigerated at 4˚C for no longer than two weeks.

Embryos were then incubated for 4 days later to record the number of four, eight-cell embryos and blastocysts respectively.

Determination of cell numbers in embryos

To determine blastocyst cell numbers from each group, embryos were placed in drops supplemented with 1 μg/ml of propidium iodide (Sigma, Germany) at 37˚C for 20-50 seconds. There were approximately 20 embryos that were in the late blastocyst stage per group. This was followed by incubation in 5 μg/ml of bisbenzimide (Hoechst 33342, Sigma, Germany) in absolute ethanol, overnight at 4˚C. The propidium iodide stained only the nucleus of non-viable cells without an intact plasma membrane, whereas bisbenzimide stained the nucleus of both viable and non-viable cells. Hence, the trophoectoderm (TE) will be stained by both propidium iodide and bisbenzimide, whilst the intact inner cell mass (ICM) will be stained only by bisbenzimide. Embryos were mounted on microscope slides with glycerol, a cover-slip was placed on the top of the embryos, and they were initially examined to evaluate the number of cells. Under fluorescence microscopy (excitation filter at 420 nm, barrier filter at 365 nm), the outer TE cells were identified by the pink fluorescence of propidium iodide, whereas the ICM cells were recognized by the blue fluorescence of bisbenzimide. The numbers of ICM and TE nuclei were counted under an inverted fluorescence microscope (IX71, Olympus, Japan).

Blastocyst development following embryo transfer

To assess the ability of late blastocysts to implant and develop *in vivo*, embryos were transferred to recipient mice. Female mice (C57BL/6, Razi Institute, Iran) were mated with a vasectomized male (C57BL/6) to produce pseudopregnant mice as recipients for embryo transfer. To ensure that all fetuses in the pseudopregnant mice were derived from embryo transfer (NMRI mouse) and not fertilized by black color mouse, we examined the skin color day 18 post-transfer.

In the control group, six blastocysts were randomly assigned to each uterine horn following developmental assessment during the *in vitro* culture. A total of 24 embryos from the control group were transferred to 4 recipients. On day 18 of pregnancy, the percentage of implantations was assessed.

Eight blastocysts from the group treated with 100 nM of melatonin were randomly assigned to each uterine horn following developmental assessment during *in vitro* culture. A total of 32 embryos were transferred per treatment to 4 recipients (Table 1). On day 18 of pregnancy, the percentage of implantations was assessed. The pregnancy rate was assayed in the embryos treated with the best dose of melatonin (100 nM), as determined by the differential staining assay, and the control group. In this study, better quality embryos were from the 100 nM melatonin-treated group.

Statistical analyses

The outcomes of the development rate were assessed using the chi-square test. Pregnancy rate and fetal weight were assessed with Fisher's exact test. All statistical analyses were performed using the Statistical Package for the Social Sciences version 16.0 for Windows. Differences were analyzed in cleavage rate, blastocyst and hatching blastocyst development rate, quality of blastocyst, and implantation outcomes, with a significance level of 0.05.

Results

In this study, 651 embryos at the two-cell stage were randomly cultured in six experimental and control groups (Table 2). When treated with 10 and 100 nM of melatonin, the rates of cleavage significantly increased compared to the control group. Our results indicated that the percent of morula formation was significantly higher in groups treated with 10 nM (94.24%) and 100 nM (91.24%) melatonin compared to the control group (80.64%, $p<0.01$). According to the results, the percentage of two-cell block decreased in groups treated with 10 and 100 nM of melatonin. The rate of blastocyst development significantly increased in the 10 nM (86.56%, $p<0.01$) and 100 nM (91%; $p<0.001$) melatonin groups compared to the control group (73.11%, $p<0.01$).

There was a significant increase in hatching percentage of embryos that were cultured in medium treated with 10 nM (61.46%, $p<0.001$) and 100 nM (58.2%, $p<0.01$) melatonin compared to the control group (43.01%, $p<0.01$).

Absolute ethanol was used to dilute melatonin (0.1% as the vehicle group) and had no detrimental effects on the cleavage and development rates, and quality of embryos ($p>0.05$).

Table 1: Effect of different doses of melatonin on mouse embryonic development in comparison to the control group

Groups	Number of recipient mice	Numberof embryos	Number of pregnancies (number of embryos)	Fetal weight (mg)
Control	4	24	2 (1 and 3)	565 ± 77
100 nM of melatonin	4	32	3 (5, 6 and 6*)	622 ± 66*

; p<0.05 vs. control group.

Table 2: Effect of different doses of melatonin on mouse embryonic development in comparison to the control group

Experimental groups	Ethanol concentrations (%)	Doses of melatonin	Numbers of two-cell	Numbers of morula (%)	Numbers of blastocysts (%)	Numbers of hatching blastocysts (%)
Control	0	0	93	75 (80.65)	68 (73.11)	40 (43.01)
Vehicle	0.1	0	97	77 (79.38)	72 (74.22)	41 (42.26)
Group 1	0.1	100 μM	96	78 (81.25)	73 (73.95)	45 (46.87)
Group 2	0.1	10 μM	108	90 (83.33)	81 (75)	52 (48.14)
Group 3	0.1	1 μM	111	92 (82.88)	86 (77.47)	53 (47.74)
Group 4	0.1	100 nM	134	123 (91.7)**	116 (86.56)**	78 (58.2)**
Group 5	0.1	10 nM	109	103 (94.4)**	99 (90.82)***	67 (61.46)***

; p<0.01 and ***; p<0.001 vs. the untreated control group.

Differential blastocyst staining

Blastocyst quality was promoted among the 10 and 100 nM melatonin-treated groups in comparison to untreated embryos (Fig 1). The total cell number (TCN), TE, and ICM of blastocysts treated with 10 and 100 nM of melatonin were higher compared to the control group (Fig 1). The mean TCN ± SD in the *in vitro* cultured blastocysts derived from two-cell embryos treated with 10 and 100 nM of melatonin were 109.6 ± 6.78 ($p<0.01$) and 121.4 ± 8.32 ($p<0.001$). The mean TE cells in these groups were 59.54 ± 5.98 and 61.64 ± 6.7 ($p<0.05$) and the TE cells were 50.06 ± 4.86 ($p<0.01$) and 59.76 ± 5.9 ($p<0.001$), respectively. The ICM: TCN percent was significantly higher in blastocysts treated with 100 nM (49.5%) compared to the control group (41.8%; $p<0.01$; Fig 1).

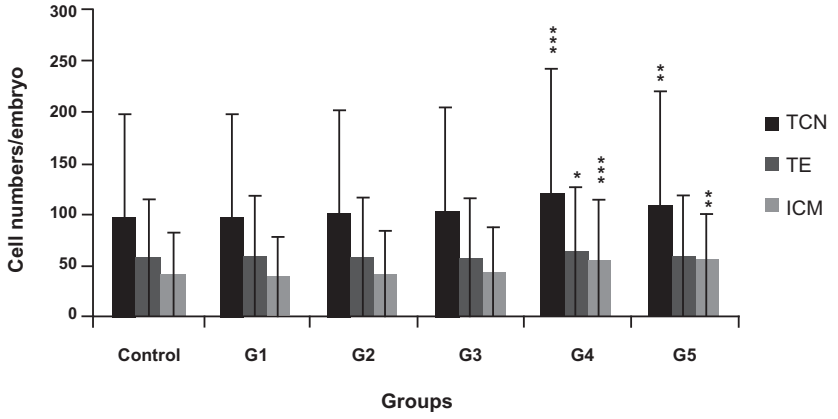

Fig 1: Effect of melatonin on embryonic cell number during IVP of two-cell embryos compared with the control group. Embryos were cultured in IVP medium that contained melatonin (G1: 100 µM, G2: 10 µM, G3: 1 µM, G4: 100 nM and G5: 10 nM). TCN in blastocysts, TE, ICM.
****; p<0.05, **; p<0.01, and ***; p<0.001 vs. the untreated control group.***

Melatonin and pregnancy outcomes

Only embryos developed in the presence of 100 nM of melatonin were transferred to pseudopregnant recipients (Table 1). In the control group, there were two pregnancies, one that had one embryo and one with three. In the treatment groups there were 5, 6, and 6 embryos (these transfers were repeated 3 times for treatment group with 100 nM of melatonin). There was a difference between the control and treatment groups in the percentage of transferred embryos that were successfully implanted ($p<0.05$). The fetal weight was also higher in the treated group (622 ± 66 mg) than the control group (565 ± 77 mg; $p<0.05$).

Discussion

We found that melatonin improved the development rate of mouse two-cell embryos when added at the two-cell stage. The rate of development to blastocyst was also significantly higher when embryos were cultured in T6 medium that contained melatonin. Ishizauka et al. (2) reported that the development rate of mouse embryos increased when embryos were cultured in BMOC-3 medium and melatonin was added 4 hours after insemination. In this study, embryos were cultured in T6 medium and melatonin was dissolved in ethanol. We added melatonin to the culture medium at the two-cell stage, when the embryos might have faced a two-cell block. Furthermore, embryo development was assessed after *in vitro* culture of the two-cell embryos. The present data has demonstrated that melatonin decreased the two-cell block and increased the blastulation rate. Our results have shown the concentration dependent effects of melatonin on embryonic development *in vitro*. Thus, melatonin may be involved in metabolism at certain stages during embryogenesis to stimulate the formation of blastocysts. Reactive oxygen species (ROS) are involved in the two-cell block phenomenon in mice (2) and melatonin is an effective ROS scavenger (9).

Embryos face the risk of exposure to high levels of ROS during *in vitro* conditions. For example, oocyte aspiration, fertilization, and embryo culture could generate higher amounts of free radicals that negatively impact early embryonic development (1). To our knowledge, the present study is the first to report on the use of melatonin in the culture of mouse embryos at the two-cell stage using T6 culture medium. We have observed that melatonin promoted TCN, TE, and ICM at 10 and 100 nM concentrations, and also had a marked positive effect on cleavage and blastocyst rates. These results agreed with previous studies in that melatonin at a concentration of 10^{-9} M increased embryo development in bovines (10) and at 10^{-6} to 10^{-8} M in mice (2).

When the number of cells in the ICM of a blas-

tocyst is decreased by approximately 30% or more, there is a high risk of fetal loss or developmental injury (11). The ICM cell number is also important for proper implantation, and thus a low ICM cell number may reduce embryonic viability (11). The TE cell also plays an important role in forming the placenta, and is required for mammalian cenceptus development (12). Reduction of TE cells causes embryonic viability and implantation suppression (13). Based on these observations, mouse blastocysts derived from two-cell embryos treated with 10 and 100 nM melatonin have resulted in increased TCN, TE, and ICM cell numbers. ICM and TCN also positively correlated with embryonic development during an embryo transfer assay (11). Our results showed that embryos treated with 100 nM of melatonin increased the implantation rate and embryo weight.

The researches of many scientists have been promoted culture systems of oocytes and embryos. For example, supplemented maturation medium with all-trans retinoic acid improved fertilization and development rates in a dose dependent manner (14). Culture in synthetic oviductal fluid promoted the potency of embryos to develop into blastocysts (15).

Physiological melatonin concentrations in the human blood are considered to be in the range of 100 pM to 1 nM (16), our results demonstrate that melatonin improves early embryonic development at physiological concentrations *in vitro*. In contrast, the presence of high melatonin concentrations (100 μM) have shown a decreased embryo development rate and inhibitory effect on the ICM/TCN ratio. Therefore, melatonin has a concentration-dependent effect on embryonic development, TCN, TE, ICM, and implantation rate.

Conclusion

Results obtained in this study indicated that the addition of melatonin at concentrations of 10 and 100 nM promoted both the quality and the quantity of embryo development in the mouse culture system. In mice, a 100 nM supplementation of melatonin to the culture medium had a beneficial effect on the embryo development during developmental stages, TCN, TE, ICM, and implantation rate. Promotion and progression of embryonic development and the ICM/TCN ratio showed the concentration-dependent regulation pattern of melatonin in the mouse culture system.

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Original Article

Biological Activity of Recombinant Accessory Cholerae Enterotoxin (Ace) on Rabbit Ileal Loops and Antibacterial Assay

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Abstract

Objective: *Vibrio cholerae (V. cholerae)* causes a potentially lethal disease named cholera. The cholera enterotoxin (CT) is a major virulence factor of *V. cholerae*. In addition to CT, *V. cholerae* produces other putative toxins, such as the zonula occludens toxin (Zot) and accessory cholera enterotoxin (Ace). The *ace* gene is the third gene of the *V. cholerae* virulence cassette. The Ace toxin alters ion transport, causes fluid accumulation in ligated rabbit ileal loops, and is a cause of mild diarrhea. The aim of this study is the cloning and overexpression of the *ace* gene into *Escherichia coli (E. coli)* and determination of some characteristics of the recombinant Ace protein.

Materials and Methods: In this experimental study, the *ace* gene was amplified from *V. cholerae* strain 62013, then cloned in a pET28a expression vector and transformed into an *E. coli* (DH5 α) host strain. Subsequently, the recombinant vector was retransformed into *E. coli* BL21 for expression, induced by isopropythio-β-D-galctoside (IPTG) at a different concentration, and examined by SDS-PAGE and Western blot. A rabbit ileal loop experiment was conducted. Antibacterial activity of the Ace protein was assessed for *E. coli, Stapylococcus aureus (S. aureus), and Pseudomonas aeruginosa (P. aeruginosa).*

Results: The recombinant Ace protein with a molecular weight of 18 kDa (dimeric form) was expressed in *E. coli* BL21. The Ace protein showed poor staining with Coomassie blue stain, but stained efficiently with silver stain. Western blot analysis showed that the recombinant Ace protein reacted with rabbit anti-*V. cholerae* polyclonal antibody. The Ace protein had antibacterial activity at a concentration of ≥200 µg/ml and caused significant fluid accumulation in the ligated rabbit ileal loop test.

Conclusion: This study described an *E. coli* cloning and expression system *(E. coli BL21-pET-28a-ace)* for the Ace protein of *V. cholerae*. We confirmed the antibacterial properties and enterotoxin activity of the resultant recombinant Ace protein.

Keywords: *Vibrio cholerae*, Accessory Cholerae Enterotoxin, pET28a, *E. coli*

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Introduction

Vibrio cholerae (V. cholera), is a major human intestinal pathogen that causes significant morbidity and mortality in developing regions of the world. Cholera is endemic in Southern Asia and parts of Africa and Latin America, with 5 million cases annually. This disease is characterized by severe diarrhea caused by toxigenic *V. cholerae* which colonize in the small intestine (1-4).

The cholera toxin (CT) is responsible for severe

dehydration that results from diarrhea associated with *V. cholerae*.

V. cholerae produces other putative toxins, such as zonula occludens (Zot) and accessory cholera toxin (Ace). The genes encoding these toxins are located on a 4.5 kb region called the "core region" or virulence cassette, which is flanked by two copies of a repeat sequence (1, 2, 5).

The Ace toxin is an integral membrane protein that consists of 96 amino acids (9-11.3 kDa) (6, 7). This toxin increases transcellular ion transport, causing FA in ligated rabbit ileal loops (4). The predicted amino acid sequence of the Ace protein shows a striking similarity to that of a family of eukaryotic ion transporting ATPases, including the human plasma membrane calcium pump, the calcium-transporting ATPase from rat brains, and the product of the *cf* gene (3, 6). The Ace protein acts synergistically with a Ca^{2+}-dependent acetylcholine analog (carbachol) and stimulation secretion has been shown to be dependent on extracellular and intracellular Ca^{2+} (8). The Ace protein also shows a sequence similarity with a virulence protein of *Salmonella dublin*, SpvB, which is virulent in mice (6). Ace is an amphipathic molecule, which when inserted into the eukaryotic cell membrane creates an ion-permeable pore located on the 26-residue δ toxin of *Staphylococcus aureus (S. aureus)* (6, 9). This study describes cloning, overexpression of Ace toxins in *Escherichia coli (E. coli)*, and determination of some characteristics of the recombinant Ace protein.

Materials and Methods

Bacterial strains and vectors

In this experimental study, *V. cholerae* strain 62013 was obtained from Pasteur Institute of Iran. *E. coli* DH5α and BL21 (PlysS) were used for cloning and expression experiments (Invitrogen and Novagen, USA). Plasmid *pET-28a*$^+$ (Novagen) was the expression vector. Bacteria were cultured in LB broth or on agar (Merck, Germany) with or without 30 μg kanamycin/ml (Sigma, USA).

Preparation of DNA template and PCR

Genomic DNA of the *V. cholerae* strain 62013 was extracted using the Bioneer Kit (South Korea). The concentration and purity of extracted DNA were determined by spectrophotometer. Specific primers were designed according to ace gene sequences of *V. cholerae* from NCBI. The sequence of the forward primer with an endonuclease site of NdeI was 5'-GCTCCATATGCTTATGATGGACACCCTTTATGAC-3', and for the reverse primer with an endonuclease site of EcoRI, it was 5'- TAGAATTCTCATAGGTTTAACGCTCGCAGGGC-3'. The PCR reaction mixture contained 0.5 μM of each primer, 10 μl 5X prime STAR buffer, 0.2 mM of each dNTP, 2.5 U of prime STAR DNA polymerase (Takara, Japan), and 200 ng genomic DNA for a final volume of 50 μl. PCR amplification was performed with an initial denaturation at 98˚C for 4 minutes, followed by 35 cycles at 98˚C for 10 seconds, 63˚C for 15 seconds, and 72˚C for 90 seconds, with a final extension for 10 minutes at 72˚C. PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel (Fermentas, USA). The desired fragments were recovered from the gel by a PCR purification kit (Bioneer, South Korea).

Cloning, expression and purification of Ace

The PCR product and pET-28a (Novagen, United States) expression vector were digested by *NdeI* and *EcoRI* and purified from in agarose gel. The resultant fragment was ligated by T4 DNA ligase (Fermentas, USA). The recombinant pET-28a was transformed to competent *E. coli* DH5α and the transformants were selected on LB agar plates that contained 30 μg/ml kanamycin.

The selected clones were confirmed by restriction enzyme digestion and PCR, and sequenced by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers (TAG Copenhagen A/S Symbion, Denmark). The result was compared to the sequence of the *ace* gene in the database with NCBI Blast Software. The recombinant plasmids were retransformed to an *E. coli* BL21 (plysS) expression host. Several conditions for the expression were tested, such as the temperature of induction and the concentration of isopropythio-β-D-galctoside (IPTG). Bacterial cells grew in the presence of kanamycin (30 μg/ml) at 37˚C with shaking (220 rpm) until an optical density at 600 nm of 0.6-1 was reached. IPTG (Sigma, USA) was added to a final concentration of 1 mM, followed by an additional 4 hours culture period at 37˚C with vigorous shaking. Cells were

harvested by centrifugation at 10000 g for 10 minutes at 4˚C to precipitate the pellet, after which the pellet was frozen at -20˚C.

The bacterial pellets were lysed using a lysis buffer (8 M urea) until the solution cleared. After centrifugation, the supernatants were examined by SDS-PAGE to verify the expressed recombinant protein.

Ace protein was purified by Ni-NTA affinity chromatography under a combination of denaturing and native conditions by binding, washing, and eluting steps according to the manufacturer's protocol (Invitrogen). In this protocol, proteins were finally eluted in 20 mM buffer that contained imidazole, and then the eluted proteins were immediately dialyzed against PBS (pH= 7.4) for removal of imidazole. Protein concentrations were determined by Bradford and nanodrop analysis and purity by SDS-PAGE. Because Ace is an acidic protein (pI 4.26), it did not stain with standard Coomassie blue staining, thus we used silver nitrate staining according to the standard protocol (2).

Anti-Ace polyclonal antibody production

The overnight culture of *V. cholerae* 62013 (toxigenic) was exposed to formalin (1.5%) at 4˚C overnight. After washing and deformalization, the culture was heated at 65˚C for 1 hour. The lysate was injected subcutaneously into a white New Zealand rabbit that weighed about 2 kg. The injection mixture contained approximately 109 bacteria per ml of physiological serum and 1 ml complete adjuvant for the first injection, from which 0.5 ml was subcutaneously injected into the shoulder. We used incomplete adjuvant as the booster on days 14, 28, and 42. Bleeding was performed prior to each injection, and the serum was separated and stored at -20˚C until use (10).

Western blot analysis

The proteins separated by SDS-PAGE were blotted onto 0.45 µm pore size PVDF membrane (Hi-bond Amersham Biosciences, USA) by using a semidry blotter unit (Bio-rad, USA). The membrane was blocked by 3% (w/v) skim milk according to standard procedures. Rabbit polyclonal anti-*V. cholerae* serum was diluted 1:500 in phosphate-buffered saline (PBS) and 0.1% (v/v) Tween 20, then incubated for 3 hours at 4˚C with shaking. The blocked membranes were washed with PBS-Tween 20, and then incubated with affinity purified goat anti-rabbit immunoglobulin G (heavy and light chain) horseradish peroxidase (HRP) conjugate antibody (Bio-Rad), at a 1:2500 dilution in PBS-Tween20. The membranes were then washed three times with PBS-Tween 20 and developed using DAB solution (Sigma, USA) (11).

Ligated rabbit ileal loop assay

New Zealand rabbits that weighed 2-2.5 kg were starved for 48 hours before the experiments. Rabbits were anesthetized by subcutaneous injection of a mixture of ketamine (50 mg/g) and acepromazine (0.5 mg/kg), and the small intestines were tied off. A total of 1 ml (500 µg/ml) of the purified Ace protein was injected into the two intestinal segments. In this experiment, 1 ml (10^8 cfu/ml) of *V. cholerae* 62013 was the positive control and sterile PBS was the negative control. Each test was undertaken on two rabbits and all rabbits were sacrificed after 18 hours. The enterotoxic response was determined by measuring the FA ratio, which is the ratio of the volume of fluid accumulated in the intestinal loop to the length of the loop. A ratio of greater than 1.0 is indicative of a strong positive response, while a negative response is defined as a ratio of less than 0.5 (11-16).

Antibacterial assay

We used bacterial strains *S. aureus* (ATCC25923), Pseudomonas aeruginosa *(P. aeruginosa*; ATCC27853), and *E. coli* (ATCC25922) to assay the antibacterial activity of the Ace protein. Cultures of each bacteria were grown overnight in LB broth, then inoculated into fresh media and grown at 37˚C until the OD595 reached 0.6. The cells were harvested and washed with 10 mM sodium phosphate (pH=7.4), then diluted in media to obtain a final density of 10^3 cfu/ml per assay sample. Bacteria were incubated with increasing concentrations of Ace protein (0, 50, 100, and 200 µg/ml) at 37˚C for 18 hours in an incubator. The cell growth was determined by colony count (7).

Results

Amplification of ace gene and construction of pET28a-ace

The amplified ace gene produced a single 299 bp band (Fig 1). The PCR product was purified and digested with *NdeI* and *EcoRI*, then sub-

cloned into the expression vector pET28a. The result of the double enzyme digestion and PCR amplification confirmed that the ace gene was exactly inserted into the PET28a vector (Fig 2). The *ace* gene nucleotide sequence in the recombinant plasmid vector of pET28a-*ace* was consistent with that of *V. cholerae ace* as published in the gene bank. The homologies of the nucleotide sequences in the pET-32a-ace compared with the published *ace* gene sequences were 99.8%.

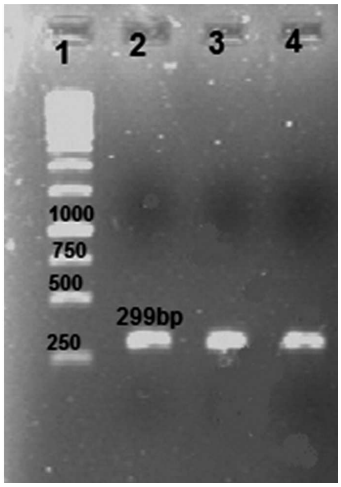

Fig 1: Electrophoresis of ace gene amplified from V. cholerae on agarose gel (1% w/v). Lane 1. 1 kb DNA size marker, lanes 2, 3, 4. single expected band of ace (approximately 299 bp).

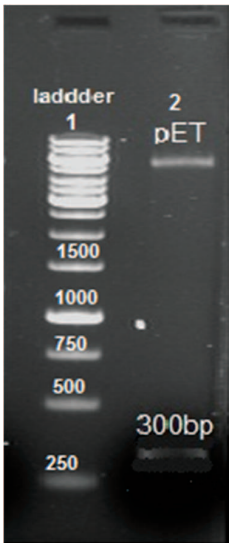

Fig 2: Agarose gel electrophoresis analysis of recombinant pET28a-ace. Lane 1. 1 kb DNA size marker, lane 2. double digestion of recombinant pET28a-ace with EcoRI, NdeI.

Expression and purification of target recombinant protein

E. coli BL21 (DE3) plysS competent cells were transformed with the confirmed recombinant vector, pET28*a-ace*. IPTG at 1 mmol/L efficiently induced the expression of *ace* fusion protein with predicated molecular masses of 18 KD (Fig 3A). Ace protein stained poorly with Coomassie blue stain, but stained efficiently with silver stain. Large scale culture and induction was performed and the resultant protein was purified by Ni^{2+} affinity chromatography under denatured and native conditions (Fig 3B).

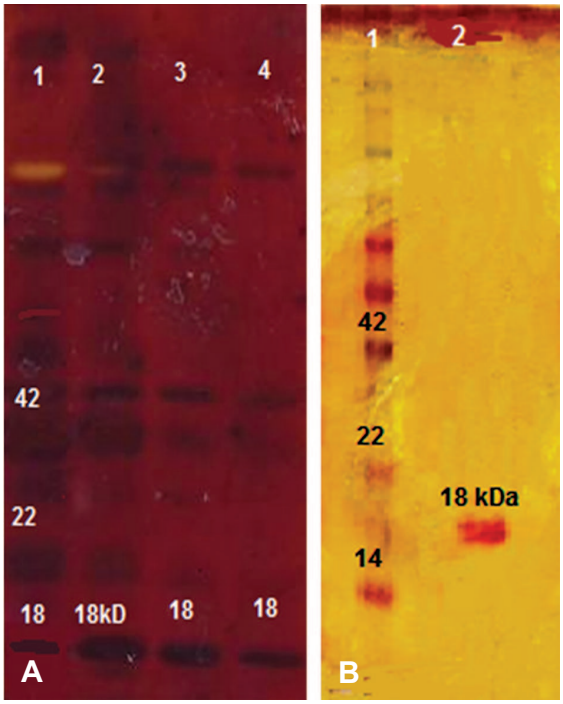

Fig 3: A. SDS-PAGE (15% w/v) analysis of expression product of pET28a-ace in E. coli BL21. Lane 1. protein marker, lanes 2, 3, 4. induction of pET28a-ace by treatment with 1mM IPTG (18 kDa). B. recombinant proteins purified by Ni-NTA column chromatograph. Lane 1. protein marker, lane 2. purified rAce protein (18kDa).

Western blot analysis

We performed Western blot analysis to detect immunogenicity of the expressed Ace protein, which was recognized by the rabbit polyclonal antibody against *V. cholerae* (Fig 4).

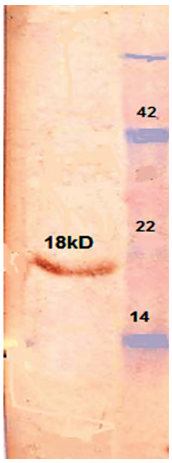

Fig 4: Western blot of the SDS-polyacrylamide gel prepared with anti-V. cholerae antibody. Antiserum was diluted 1:500. The 18 kDa proteins of the recombinant Ace were detected.

Rabbit ileal loop test with recombinant protein

The purified Ace protein (500 µg) induced significant FA (ratio: 1.25 ± 0.2) in segmented rabbit ileal loops (Fig 5). FA of PBS (negative control) was not significant (ratio: 0.5 ± 0.005), while FA of the positive control was significant (ratio: 2 ± 0.2).

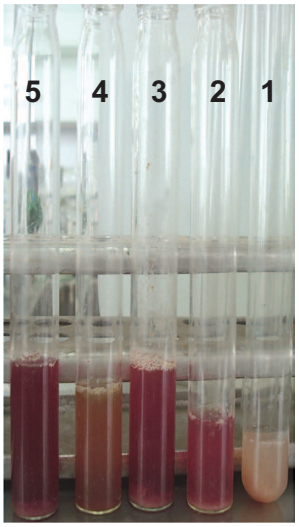

Fig 5: Rabbit ileal loop assay. Ileal tissues treated with recombinant Ace protein culture supernatants of the toxigenic V. cholerae 62013 (positive control). Sterile PBS was used as a negative control. Tube 1. fluid accumulation (FA) in negative control (ratio 0.5 ± 0.005), tube 2. significant hemorrhagic FA (ratio 1.25 ± 0.2) with recombinant Ace protein, tubes 3 and 4. other test substances, and tube 5. significant hemorrhagic FA (ratio 2 ± 0.2) with positive control.

Antibacterial assay

We tested for antibacterial activity of purified the recombinant Ace protein to S. aureus, E. coli, and *P. aeruginosa*. There was inhibition and decreased bacterial growth at the 200 µg/ml concentration of Ace protein.

Discussion

Ace is a third toxin of *V. cholerae* (1) that causes milder cholera symptoms. It may contribute to an early phase of intestinal secretion in infections by *V. cholerae*, which can occur prior to the onset of secretion stimulated by the cholera toxin (8). Non-enterotoxigenic *V. cholerae* cells that lack *ace, zot*, and ctx genes do not cause diarrhea in volunteers (14). Ace toxin increases the potential difference (PD) across the intestinal epithelium, alters ion transport, and increases the short-circuit current in rabbit ileal tissues that have been mounted in Ussing chambers (1-4, 14, 15). In this study the cloning of the *ace* gene was confirmed by colony-PCR, enzymatic digestion, and sequencing in *E. coli* BL21-pET-28a-*ace.* The nucleotide sequence of the *ace* gene in plasmid pET28a was 100% homologous with the *ace* gene reported in the gene bank (Z22569.1).

The quantity of Ace protein produced in wild type *V. cholerae* was estimated to be 0.6 mg/lit, which was 10000 fold lower than produced in our expression system (5 mg/lit). The production of the recombinant Ace protein in a concentration of 7 mg/lit in a yeast system was reported previously by Trucksis et al. (2). However, because of *E. coli*'s ability to grow rapidly and at a high density on inexpensive substrates, the prokaryotic expression system has remained very attractive for the production of recombinant proteins (15). According to other studies, expression of the recombinant Ace protein was successful in *E. coli* (LMG194), but in *E. coli* TOP10, the Ace protein was not successfully produced (1, 4).

Ace protein was purified by affinity chromatography with Ni-NTA resin. The predominant form of the Ace toxin was an 18 kD dimeric form. The Ace protein was not completely denatured and persisted as a dimer and multimer in the gel (1). The

Ace protein was detected in both monomer and dimeric forms by Trucksis et al. (2). Chatterjee et al. (7) reported only the dimeric forms of this protein. Our recombinant Ace protein did not stain with Coomassie blue, thus we used silver stain, as reported previously (1, 3, 15). The immunogenicity of this protein was confirmed by polyclonal antibody against *V. cholerae*, by enterotoxicity in rabbit ileal loops, and its antimicrobial effects showed it is active biologically. Ace protein has recently been used to treat cystic fibrosis (CF). CF involves insufficient chloride transport and loss of luminal sodium and water, leading to damage of the bronchial connective tissue. The administration of Ace increases the level of secretion of chloride in the lungs and leads to an increase in the amount of airway surface water in the lumen of the lungs (9). Therefore, recombinant production of the Ace protein can be useful for medical applications.

Conclusion

Our highly efficient expression system *(E. coli BL21- pET-28a-ace)* can be used for conducting various biological experiments and has facilitated the production of pure proteins free from other *Vibrio* antigens for investigation as potential vaccine candidates.

Acknowledgments

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